United States Department of Agriculture
Animal and Plant Health Inspection Service
Plant Protection and Quarantine

PCR Detection and DNA Isolation Methods
for Use in the *Phytophthora ramorum* National Program

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I. Survey Recommendations:

The validated protocols listed below are from Oregon State University and the University of California at Berkeley with modifications based on the work done in Beltsville by USDA APHIS PPQ CPHST. The following recommendations are provided based on our experience with DNA extraction and PCR protocols tested.

- DNA can be extracted using either the OSU DNA extraction protocol or the Qiagen DNeasy Plant Mini kit. PCR amplification was comparable using both protocols. One reason we would recommend the kit over the OSU protocol is that it is easier to use and all reagents and kit components are standardized and quality assured. We also note that the kit produces higher yields of DNA which appears to be of better quality. We make this recommendation for culture and leaf material only.
- The PCR for use in the survey should be Protocol 2 (nested PCR test) developed at UC Berkeley. This test, in combination with the DNeasy kit or the OSU, CTAB-based extraction gave excellent sensitivity (Fig. 6), where samples could be diluted to 1:50,000. We have modified the OSU protocol slightly and the modified protocol is included in this document (below).
- From limited experiments we have determined that up to 10 samples can be composite for DNA extraction and subsequent PCR.
- Any sample reacting positive in a PCR test will have to be confirmed by USDA APHIS PPQ NIS and the CPHST lab in Beltsville, MD. Any such sample is considered a suspect positive until confirmation. All suspect positives must be reported to the USDA APHIS PPQ State Plant Health Director in your state. All suspect positive cultures will be sent to the NIS laboratory in Beltsville, MD for morphological and molecular confirmation.

II. Molecular Methods: DNA Extraction

NOTE: In order to avoid cross contamination please designate separate rooms or lab areas for each segment of the work and use separate sets of pipettes. Use aerosol-resistant pipette tips. Centrifuge any DNA-containing tubes before opening so that any liquid near the rim of

the tube is removed; centrifuge rotors designed for aerosol containment are recommended. It is a good practice to store plant samples or extracts in a separate freezer or freezer compartment from PCR reaction components. If samples are contaminated with soil rinse them in sterile water and pat-dry them with a hand towel. Wear gloves and change them regularly, particularly between different procedures. Use disposable lab mats to cover bench areas and change them often. Never autoclave any *P. ramorum*–contaminated plant material, culture plates, or soil in an autoclave used to sterilize buffers, glassware, or plastic ware used in the SOD PCR because of potential contamination from aerosols within the autoclave.

A. Modified OSU DNA Extraction Protocol:

We tested this protocol only with mountain laurel infected and healthy leaf tissue. It's efficacy for tissue samples from other sources remains to be determined. All stock solutions should be prepared in advance. When processing many samples, aliquot the necessary volume of the stock solution for the number of the samples plus 2-3 more in a new tube to prepare a working solution. The extraction should be conducted in a room or an area separate from the location used to set up master mix and PCR reactions.

1. Make 2% CTAB (cetiltrimethylammonium bromide SIGMA) buffer, 100 mM Tris, pH 8.0; 20 mM Na_2EDTA pH 8.0; 1.4 M NaCl; 1% polyvinylpirolidone, 0.1% 2-mercaptoetanol [ME]). (We prepare the buffer without 2-ME. Before using it, take the necessary volume for the number of samples (600µl per sample) and add 2-mercaptoethnol to 0.1%, in a fume hood.)
2. Pre-warm CTAB buffer to 65°C.
3. Use a No. 3 cork borer (0.6 cm in diameter) to cut out leaf tissue from suspect SOD-infected samples. Sterilize cork borer after each separate sample by dipping in EtOH and flaming. After flame-sterilizing the cork borer it will not be sufficiently sharp, so you may want to use disposable blades and cut out a square of plant tissue with sides of 0.5 cm long.
4. Place the plant disc into a 2 ml screw-cap plastic tube, add two glass beads, screw the cap tightly and drop the tube into liquid nitrogen for around 30 sec.
5. Take the tube out of the liquid nitrogen and immediately place it onto Mini Beat-Beater. Beat for 30 sec at 2,500 rpm (setting 3 for time and 25 for speed). If you composite several (up to 10) samples, you will need to repeat freezing and grinding 2 more times.
6. Add 500 µl of pre-warmed CTAB buffer. Vortex.
7. Incubate for 1 hour at 65°C, mixing time to time.
8. Add 500 µl phenol: chloroform: isoamyl alcohol (25:24:1) (we do not take glass beads out at this point). Vortex for 1 min.
9. Spin on a microcentifuge for 10 min at 13,000 rpm.
10. Carefully remove the supernatant (approx. 450 µl) and transfer it into a new microcentrifuge tube. Add 450 µl chloroform: isoamyl alcohol (24:1) and vortex for 1 min.
11. Spin again as at step 9.
12. Carefully remove the supernatant and transfer it into a new microcentrifuge tube containing 200 µl of 100% EtOH (200 proof)). Mix well by inverting.
13. Pour the mix into DNeasy column (DNeasy Tissue Kit from Qiagen Cat. #69504).

14. Spin for 1 min at 8,000 rpm on microcentrifuge at room temperature (RT). Pour out the flow-through, keep the column.
15. Wash the column with 500 µl AW1 buffer (from the kit). Spin 1 min / 8,000 rpm at RT. The AW1 and AW2 buffers are provided with the kit and have to be diluted with 100% EtOH prior to use (see manufacturers instructions).
16. Transfer the column into a new collection tube and wash with 500 µl AW2 buffer. Spin for 3 min at 8,000 rpm at RT until the buffer passes through the column leaving the column dry.
17. Transfer the column into 1.5 ml microcentrifuge tube (you may need to cut off the lid to fit into the microcentrifuge).
18. Add 50 to 100 µl of the Elution Buffer exactly on the top of the filter column, not the walls. Incubate at RT for 1 min.
19. Spin for 1 min at 8,000 rpm at RT.
20. If you have cut off tube lids transfer resulting DNA in a new tube, label and keep DNA at -20ºC.

B. Plant DNA Extraction Using Qiagen DNeasy Plant Mini Kit (Cat # 69104) (Modified for composite samples)

1. Pre-warm AE buffer to 65ºC in water-bath or thermostat.
2. Use cork borer No 3 (0.6 cm in diameter) to cut out leaf tissue from suspect SOD-infected samples. Sterilize cork borer after each separate sample by dipping in EtOH and flaming. After several rounds of flaming cork borer won't be sufficiently sharp anymore so you may want to use disposable blades and cut out a square of plant tissue with sides of 0.5 cm long. Max weight of tissue to be used for DNA extraction with the kit is 100 mg.
3. Place a glass bead (5mm diameter) in a 2 ml screw-cap tube then add 10 pieces of tissue (could be 10 different samples) then another glass bead. Tightly screw tube cap and drop the tube in liquid nitrogen for about 30 sec. Take tube out and place it immediately on a Bead-Beater. Homogenize for 30 sec at 2500 rpm (settings 3 for time and 25 for speed). Remove the tube and place it in liquid N_2 again for 30 sec. Repeat freezing and beating 2 more times. Place tube in ice while homogenizing next sample. Alternatively you can use mortar and pestle to grind samples.
4. Add 600µl of buffer AP1 and 6µl of RNase A to each tube, mix well by inverting and incubate at 65ºC for 15 min. Mix tubes time to time by inverting.
5. Add 195µl of buffer AP2 to each sample, mix well by inverting and incubate on ice for 5 min.
6. Centrifuge samples on micro centrifuge for 5 min at full speed (usually 10,000 rpm).
7. Transfer supernatants to QIAshredder spin columns (lilac colored tubes) placed in a 2ml collection tubes and centrifuge for 2 min at max speed.
8. Transfer 450 µl of the flow-through fraction (try not to disturb pellets formed on the bottom of the tubes) to a new 1.5 ml tube and add 1.5 volumes of AP3/E buffer (675 µl) and mix well by pipetting. If you recover less or more than 450 µl calculate how much AP3/E buffer you need to add.
9. Follow protocol provided with the kit.
10. To elute DNA use 100 µl of pre-warmed AE buffer. Don't do second elution.

11. Label tubes and keep extracted DNA at -20ºC.
12. For PCR, dilute DNA 1:10 in sterile 0.1X TE buffer pH 8.0.

III. Molecular Methods: PCR Amplification

Modified UCB DNA Amplification (PCR) Procedure:

All reagents should be kept at -20°C. Before PCR take them out to thaw, vortex briefly and spin down shortly to collect the content on the bottom. While preparing the master mix, all reagents should be kept on ice. The master mix should be prepared in a laminar flow hood, or in a room where PCR products are not generated or analyzed. Use a separate set of pipettes for the master mix. Change tips and gloves often.

The PCR mix for the first round of amplification (using specific PCR primers Phyto 1 and Phyto 4) consists of:

10X PCR Buffer	2.50	µl	
10mM dNTPs	0.50	µl	
50mM $MgCl_2$	1.00	µl	
5µM Phyto 1/4primer mix	2.50	µl	(0.5µM final concentration)
Platinum Taq polymerase (5u/µl)	0.25	µl	
dH_2O	12.00	µl	
Total mix volume	18.75	µl	
Add DNA	6.25	µl	
Total reaction volume	25.00	µl	

Prepare **master mix** for the number of the samples tested, plus 1-2 extra and keep on ice. Don't forget to include positive and negative (water used to make the master mix and 0.1X TE buffer used for DNA dilutions) controls. Aliquot 18.75µl of the master mix to each PCR tube. Take tubes to the PCR station and add 6.25 µl of each DNA sample with a pipette used only for this purpose. No mineral oil needed if you run PCR on thermocycler with heated lid.

First round PCR program:

Denaturation:	94°C / 1min 25 sec
1 cycle	
Amplification:	93°C / 35 sec
34 cycles	62°C / 55 sec
	72°C/ 50 sec
Extension:	72°C/ 10 min
1 cycle	
Hold:	4°C

Ramp rate: 3.3°C/ sec heating and 2.0°C/ sec cooling (if possible).

Take special care while preparing diluted template samples for the second round of PCR in order to avoid cross contamination.
In a laminar hood or PCR set up area prepare Eppendorf tubes with 500 µl sterile distilled H2O for the number of the samples you are running. Take those tubes to the PCR station/area and add 1 µl of corresponding first round PCR reactions to make 1:500 dilutions. **Close tubes tightly, vortex and spin down briefly**. Change gloves between opening the PCR tubes and tubes with diluted templates –first round products. Be careful to avoid any aerosol formation.
In the laminar hood make up the PCR mix for the **second round** of the nested PCR.

The PCR mix for the second round of amplification (using Universal PCR primers Phyto 2 and Phyto 3) consists of:

10X PCR Buffer	2.50 µl	
10mM dNTPs	0.50 µl	
50mM $MgCl_2$	1.00 µl	
5µM Phyto 2/3 primer mix	2.50 µl	(0.5µM final concentration)
Platinum Taq polymerase (5u/µl)	0.25 µl	
dH_2O	12.00 µl	
Total volume	18.75 µl	
Add DNA	6.25 µl	
Total reaction volume	25.00 µl	

Dispense 18.75 µl of the master mix to each tube and take tubes to the PCR station. Add 6.25 µl of each diluted first-round sample, mix and place on the block. Include new controls: sterile water used for dilution, water used for master mix and positive control (diluted first round product).

Second round PCR program:

Denaturation:	94°C / 1min 25 sec
1 cycle	
Amplification:	93°C / 35 sec
34 cycles	62°C / 55 sec
	72°C/ 50 sec
Extension:	72°C/ 10 min
1 cycle	
Hold:	4°C

Ramp rate: 3.3°C/ sec heating and 2.0°C/ sec cooling (if possible).

To visualize PCR results run 10 µl of each PCR sample, from the second round only on 1.5% agarose gel in 1X TAE buffer at 100V for 1 hour. Stain gels with the EtBr according to you lab protocol.

IV. Equipment and Reagents Necessary for SOD Detection:

Protocol 1 PCR Primers and Reagents

A. PCR machine - we use T-gradient from Biometra (others could be used).
B. Primers:
    a. Specific *P. ramorum* primer pair:
        i. Phyto 1: 5'-CAT GGC GAG CGC TTG A-3'
        ii. Phyto 4: 5'-GAA GCC GCC AAC ACA AG-3'
    b. *Phytophthora* universal primer pair:
        i. Phyto2: 5'-AAA GCC AAG CCC TGC AC-3'
        ii. Phyto3: 5'-GGT GGA TGG GGA CGT G- 3'
    c. We ordered primers desalted from Invitrogen (you can use your usual provider) and prepare primer mixes the following way:
        i. Tubes with lyophilized primers were spun briefly before opening the tubes. Primers were rehydrated to 100 µM concentration in autoclaved
        ii. 0.1 X TE buffer pH 8.0 and stored at - 20°C.
        iii. 50 µM stocks of primer mixes of the specific pair (Phyto 1 and 4) and the control pair (Phyto 2 and 3) were made.
        iv. 5 µM working mixes were prepared by diluting an aliquot of the 50 µM stocks 1:10 in 0.1 X TE buffer pH 8.0.
C. Reagents
    a. Enzyme– Platinum *Taq* Polymerase from Invitrogen (cat. No 10966-034). Supplied as a kit with 10X PCR Buffer and 50mM $MgCl_2$.
    b. 10mM **dNTP Mix** from Sigma (D-7295).
    c. **Thin-wall 0.2ml PCR** tubes from Cole-Parmer (No. 67103-90).
    d. Molecular grade d H_2O from Geno Technology Inc. (1-800-628-7730).
    e. Sterile 0.1X TE buffer pH 8.0

f. Sterile distilled water.

OSU DNA Extraction Protocol

1. Reagents for CTAB buffer (2% CTAB (cetyltrimethylammonium bromide), 100mM Tris, pH 8.0; 20mM Na2EDTA pH 8.0; 1.4M NaCl; 1% polyvinylpirolidone, 0.1% 2-mercaptoetanol).
2. Phenol: chloroform: isoamyl alcohol (25:24:1) from Invitrogen Cat # 15593-031.
3. 200 Proof (100%) Ethanol.
4. DNeasy Tissue Kit from Qiagen Cat. # 69504.
5. Screw-cap plastic tubes – 2 ml volume.
6. Glass beads 5mm diameter from Fisher Scientific cat. # 11-312C.
7. Mini Bead-beater (Cole Parmer, Cat# A-36270-02, ~$800.00, 1800-323-4340)

Qiagen DNeasy Plant extraction protocol

1. DNeasy Plant Mini Kit from Qiagen Cat # 69104.
2. Glass beads 5mm diameter from Fisher Scientific cat. # 11-312C.

Vendors:

- **Cole Parmer (800-323-4340)**
- **Qiagen (800-426-8157)**
- **Invitrogen (800-955-6288)**
- **Geno Technology Inc. (800-628-7730)**
- **Sigma (800-325-3010)**
- **Biospec Products, Inc (800-617-3363)**
- **Fisher Scientific (800-766-7000)**
- **LabRepco (800-521-0754)**